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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Identification of pig liver esterase variants by tandem mass spectroscopy analysis and their characterization

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Abstract Pig liver esterase (PLE) is probably the most important carboxyl esterase in organic synthesis and is commercially obtained by extraction of the animal tissue. However, problems occur in its application due to the presence of several isoenzymes (α-, β- and γ-PLE). The functional expression of the γ-isoenzyme was already shown and differences in the enantioselectivity compared to the commercial preparations were confirmed. The amino acid and nucleotide sequences of the α- and β-PLE are still unknown. In this work, putative sequences of the α-isoenzyme were identified from a commercial PLE preparation by 2D gel electrophoresis, digestion with proteases and analysis using Matrix-assisted laser desorption/ionization–time of flight (TOF) and electrospray ionisation quadrupole–TOF mass spectrometry. Based on these results, three amino acid exchanges were introduced into the gene encoding γ-rPLE by site-directed mutagenesis, and the proteins were expressed in *E. coli* Origami (DE3). The produced PLE mutants were characterised with respect to their substrate specificity and enantioselectivity. No significant differences in the activity towards methyl butyrate were found, but several variants showed substantially enhanced enantioselectivity in the resolution of (*R*,*S*)-1-phenyl-2-butyl acetate with $E=100$ for the best mutant V236P/A237G.

Keywords Enantioselectivity · Isoenzymes · MALDI-TOF · Pig liver esterase

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Introduction

Pig liver esterase (PLE; EC 3.1.1.1) is the most important carboxylesterase used as catalyst in organic synthesis to produce optically active compounds (Faber 2004; Bornscheuer and Kazlauskas 2006). However, in using commercial PLE preparations obtained by extraction of pig liver tissue often irreproducible results are obtained, as these are ill-defined compositions of PLE isoenzymes (α, β and γ-PLE). It could be shown that these isoenzymes differ by isoelectric point, molecular weight, sensitivity towards inhibitors and, most importantly, substrate specificity (Heymann and Junge 1979a, b; Farb and Jencks 1980; Öhrner et al. 1990; Barker and Jencks 1969). In comparison to the γ-PLE converting more selectively proline-β-naphthylamide, the α-isoenzyme preferentially uses methyl butyrate as substrate. These properties enable to distinguish between the isoenzymes. However, it is unknown how the isoenzymes differ on the amino acid level.

Several years ago, we determined the N terminal amino acid sequence of a commercial PLE sample and found that this showed high homology to a published sequence encoding a proline-β-naphthylamidase (Takahashi et al. 1989; Heymann and Peter 1993). Next, we successfully

isolated the gene encoding this enzyme from cDNA derived from pig liver mRNA by reverse transcription-polymerase chain reaction (RT-PCR) and functionally expressed the gene in the yeast *Pichia pastoris* (Lange et al. 2001) and, very recently, also in *Escherichia coli* (Böttcher et al. 2007). We could confirm by biochemical characterisation that the proline-β-naphthylamidase indeed resembles the γ-isoenzyme of PLE, which also showed significantly higher enantioselectivity towards several acetates of secondary alcohols compared to a commercial (crude) PLE sample (Musidlowska et al. 2001). Thus, it is possible to produce the γ-PLE recombinantly, preventing the problems associated with inhomogenous commercial PLE preparations obtained by simple extraction from animal tissue.

Furthermore, the closely related porcine intestinal carboxylesterase differing by 17 amino acids from γ-PLE was created by site-directed mutagenesis from the gene encoding γ-PLE. Again, all mutants exhibited different substrate specificities and enantioselectivities (Musidlowska-Persson and Bornscheuer 2003a, b).

However, the amino acid and nucleotide sequences of α- and β-PLE are still unknown. To overcome this limitation, we aimed in this investigation at the identification of these amino acid sequences by mass spectrometry analysis. The isoenzymes from a commercial PLE preparation were separated by 2D gel electrophoresis, digested with proteases and analysed by Matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) and electron spray ionisation–quadrupole (ESI-Q-TOF) mass spectrometry.

The identified differences in the amino acid sequences were introduced by site-directed mutagenesis into the recombinant γ-rPLE and expressed in *E. coli* Origami (DE3). The produced variants were characterised with respect to their substrate specificity and enantioselectivity.

Materials and methods

All chemicals were purchased from Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Promega (Mannheim, Germany) at the highest purity available. (*R*,*S*)-1-Phenyl-1-ethylacetate (**1**) was from Fluka, acetates **2**–**5** ((*R*,*S*)-1-phenyl-2-butylacetate (**2**), (*R*,*S*)-1-phenyl-2-propylacetate (**3**), (*R*,*S*)-1-phenyl-1-propylacetate (**4**), (*R*,*S*)-4-phenyl-2-butylacetate (**5**)) were synthesised from commercially available alcohols using standard procedures as already described (Musidlowska-Persson and Bornscheuer 2002). Oligonucleotides and DNA sequencing were obtained from MWG-Biotech (Ebersberg, Germany). The QIAprep Spin Miniprep kit and PCR purification kit (Qiagen, Hilden, Germany) were used for DNA purification. The antibiotic ampicillin was purchased from Roth (Karlsruhe, Germany), and the restriction- and DNA-modifying enzymes from New England Biolabs (Ipswich, MA, USA) and Promega. Proteases were obtained from Sigma and Promega and the commercial PLE preparation from Fluka.

Microorganisms, plasmids and growth condition

The vector pET-15b (Novagen, San Diego, CA, USA) was used for cloning and mutation experiments in *E. coli* DH5α [supE44ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1; Clontech, Mountain View, CA, USA] and for the recombinant expression in *E. coli* Origami (DE3) [Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araΔ139 ahpC galE galK rpsL F′[lac+ lacIq pro] (DE3) gor522::Tn10 trxB; KanR, StrR, TetR; Novagen, San Diego, CA, USA]. For coexpression experiments, the Chaperone plasmid set (TAKARA BIO, Otsu, Shiga, Japan) containing the plasmid pGro7 was used. Cells were cultivated in Luria–Bertani medium [yeast extract (5 g/l), peptone (10 g/l) and NaCl (10 g/l)] supplemented with the appropriate antibiotics at 30°C.

2D gel electrophoresis and mass spectrometry

Separation of the proteins was carried out in the first dimension by an isoelectric focusing (IPGphor, Pharmacia Biotech) and in the second dimension by a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). For isoelectric focusing, 50 μg Fluka-PLE was absorbed on gel strips, which carried an immobilised pH gradient (Immobiline™ Dry Strip pH 4–7; Amersham Biosciences, Uppsala, Sweden).

The protein spots were excised from stained 2D gels using a Ettan spot cutter (Amersham Biosciences) with a picker head of 2-mm diameter. Cut spots were transferred into 96-well microtiter plates. The tryptic digest with subsequent spotting on a MALDI-target was carried out automatically with the Ettan spot handling workstation (Amersham Biosciences).

Additionally, in-gel tryptic (Promega), AspN (Sigma) or GluC (Sigma) digestion were also performed manually, and sample template preparation for MALDI-TOF MS (MALDI-TOF PA 4700, Applied Biosystems, Foster City, CA, USA) or high-performance liquid chromatography (HPLC)–ESI-qQ-TOF MS (HPLC Dionex LC-Packings, Amsterdam; ESI-qQ-TOF Qstar Pulsar, Applied Biosystems) was carried out by mixing 0.5 μl of the resulting peptide solution with an equal volume of saturated α-cyano-4-hydroxy cinnamic acid solution in 50% (*v*/*v*) acetonitrile and 0.1% (*w*/*v*) trifluoroacetic acid.

The MALDI-TOF measurement was carried out on the 4700 MALDI TOF/TOF analyzer (Applied Biosystems).

This instrument is designed for high throughput measurement, being automatically able to measure the samples, calibrate the spectra and analyse the data using the 4000 Series Explorer™ Software V3.5.1.

The spectra were recorded in a mass range from 900 to 3,700 Da with a focus mass of 2,000 Da. For one main spectrum, 30 sub-spectra with 60 shots per sub-spectrum were accumulated using a random search pattern. If the autolytical fragments of trypsin with the mono-isotopic $(M+H)^+$ m/z at 1,045.556 and 2,211.104 reached a signal to noise ratio (S/N) of at least 20, an internal calibration was automatically performed as two-point calibration using these peaks. The standard mass deviation was less than 0.15 Da. If the automatic mode failed (in less then 1%), the calibration was carried out manually.

After calibration, the peak lists were created using the "peak to mascot" script of the 4000 Series Explorer™ Software. Settings were a mass range from 900 to 3,800 Da, a peak density of 15 peaks per 200 Da, a minimal area of 100 and maximal 60 peaks per spot. The peak list was created for a signal to noise (S/N) ratio of 10.

The MALDI-TOF/TOF measurements were carried out on the 4700 MALDI TOF/TOF analyzer (Applied Biosystems).

From the TOF spectra, the two strongest peaks were analysed. For one main spectrum, 25 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point calibration with the mono-isotopic arginine $(M+H)^+$ m/z at 175.119 or lysine $(M+H)^+$ m/z at 147.107 to reach a S/N ratio of at least 5.

The peak lists were created using the "peak to mascot" script of the 4000 Series Explorer™ Software. Settings were a mass range from 60 to precursor, 20 Da, a peak density of 15 peaks per 200 Da, a minimal area of 100 and maximal 65 peaks per precursor. The peak list was created for a S/N ratio of 7.

For database search, the Mascot search engine version 2.1 (Matrix Science, London, UK) with a specific *NCBInr* sequence database was used.

Site-directed mutagenesis

To introduce specific mutations, the QuikChange site-directed mutagenesis method (Stratagene, San Diego, USA) was applied. As template, the plasmid pET-15b-mPLEtag (7,343 bp) harbouring the γ-PLE gene without the natural signal sequence and without the terminal tetrapeptide HAEL (Lange et al. 2001; Böttcher et al. 2007) was used. Cloning into the vector pET-15b allowed an N terminal fusion of the PLE gene to a His-tag sequence, making metal affinity chromatography purification of the protein possible.

The mutated plasmids were transformed into *E. coli* DH5α and, after sequencing, into *E. coli* Origami (DE3).

The following mutagenesis primers were used:

QC-Q294P	FW 5′-TTGATTTTCATGGAGACCCA AGAGAGAGCCATCCC-3′
	RV 5′-GGGATGGCTCTCTCTTGGGT CTCCATGAAAATCAA-3′
QC-K463N	FW 5′-TTGGTTTTCCACTGTTAAAC GGCGATGCCCCAG-3′
	RV 5′-CTGGGGCATCGCCGTTTAAC AGTGGAAAACCAA-3′
QC-P302T	FW 5′-CCATCCCTTCCTGACCACTG TGGTGGATG-3′
	RV 5′-CATCCACCACAGTGGTCAGG AAGGGATGG-3′
QC-V236/A237	FW 5′-GTGGCCCTCACTNNTNNCCT GGTCAGGAAGGACATG-3′
	RV 5′-CATGTCCTTCCTGACCAGGN NANNAGTGAGGGCCAC-3′

Coexpression of the PLE variants with chaperones

The expression of the enzymes was carried out by coexpression of the chaperones GroEL/GroES in *E. coli* Origami (DE3) according to Böttcher et al. (2007) using 0.5 mg/ml L-arabinose for the induction of the chaperone expression and 40 μM IPTG for the induction of the PLE expression.

SDS–polyacrylamide gel electrophoresis

Commercial pig liver esterase solutions were separated on polyacrylamide gels (12.5%) according to Laemmli (Laemmli 1970) and stained for protein detection with Coomassie Brilliant Blue R250.

Determination of esterase activity

Esterase activity was determined spectrophotometrically in sodium phosphate buffer (50 mM, pH 7.5) using *p*-nitrophenyl acetate (10 mM in DMSO) as substrate. The amount of *p*-nitrophenol released was routinely determined at 410 nm ($\varepsilon = 14.76 \times 10^3\ M^{-1}\ cm^{-1}$) and room temperature. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under assay conditions.

Substrate specificity of PLE was measured using a pH-stat assay. To an emulsion (30 ml) containing methyl butyrate [5% (*v*/*v*)] and gum arabic [2% (*w*/*v*)], a known amount of esterase was added at 37°C. Liberated acid was titrated automatically in a pH-stat (Schott, Mainz, Germany) with 0.01 N NaOH to maintain the pH constant at 7.5. One

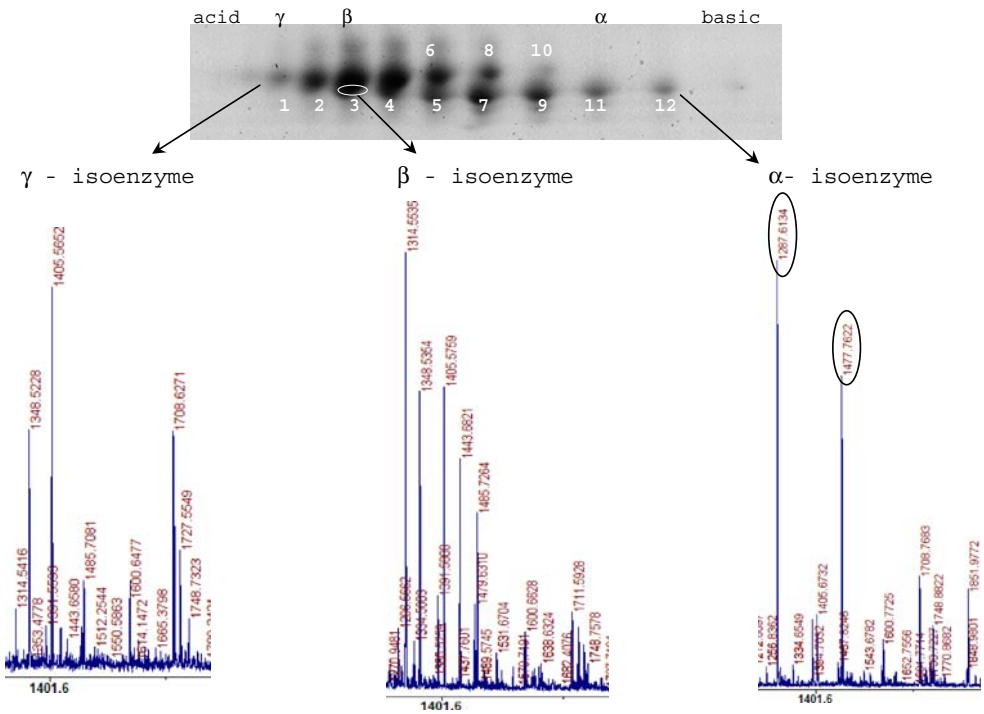

Fig. 1 Separation of a commercial PLE preparation (Fluka) on a 2D gel and corresponding mass spectra

unit of activity was defined as the amount of enzyme releasing 1 μmol acid per minute under assay conditions.

Esterase-catalysed kinetic resolution of acetates **1**–**5**

Racemic acetates **1**–**5** were dissolved in sodium phosphate buffer (50 mM, pH 7.5) to a final concentration of 10 mM. Kinetic resolutions were started by addition of 0.5 U (based on the pNPA assay) esterase to 1 ml of substrate solution. The hydrolysis reactions were carried out in a thermomixer (Eppendorf, Hamburg, Deutschland) at 37°C. To terminate the reactions, the mixtures were extracted with methylene chloride, and the organic phases were dried over anhydrous sodium sulfate. The determination of enantiomeric purity and conversion was performed by gas chromatography as described (Musidlowska-Persson and Bornscheuer 2002). The enantioselectivity (*E* value) was calculated according to Chen et al. (1982).

Creation of homology models

The 3D structure of PLE was modelled based on the known structure of human liver carboxylesterase I [PDB entry: 1MX9A (Bencharit et al. 2003)] using Pymol, an automated

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  1 gqpasppvvd taqgrvlgky vsleglaqpv avflgvpfak pplgslrfap pqpaepwsfv
 61 knttsyppmc cqdpvveqmt sdlftngker ltlefsedcl ylniytpadl tkrgrlpvmv
121 wihggglvlg gapmydgvvl aahenvvvva iqyrlgiwgf fstgdehsrg nwghldqvaa
181 lhwvqenian fggdpgsvti fgesaggesv svlvlsplak nlfhraises gvaltvalvr
241 kdmkaaakqi avlagckttt savfvhclrq ksedelldlt lkmkfltldf hgdqreshpf
301 lptvvdgvll pkmpeeilae kdfntvpyiv ginkqefgwl lptmmgfpls egkldqktat
361 sllwksypia nipeeltpva tdkylggtdd pvkkkdlfld lmgdvvfgvp svtvarqhrd
421 agaptymyef qyrpsfssdk kpktvigdhg deifsvfgfp llkgdapeee vslsktvmkf
481 wanfarsgnp ngeglphwpm ydqeegylqi gvntqaakrl kgeevafwnd llskeaakkp
541 pkikhael
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Fig. 2 Amino acid sequence of the γ-PLE ("proline-β-naphthylamidase", AC-nr 1802273A; without signal sequence): active carboxylesterase (1–544), ER retention signal (545–548), amino acids of the active site (*bold*), conserved regions (*grey background*), peptides missing in the α-fraction (*underlined*)

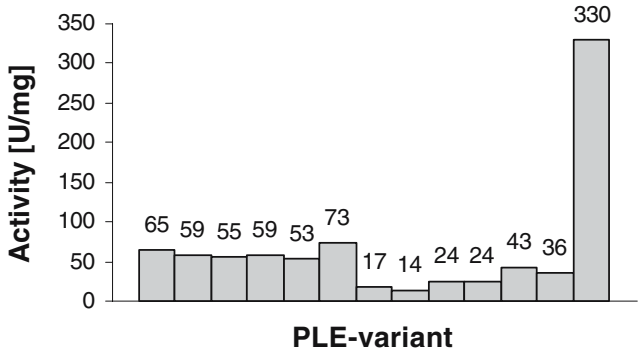

Fig. 3 Volumetric activities of PLE mutants A-G, W1-W4, γ-rPLE and Fluka-PLE (from *left* to *right*) towards methyl butyrate as determined in the pH-stat assay

protein structure homology-modelling server available at http://pymol.sourceforge.net/.

Results

For the identification of the amino acid sequences of the α- and β-isoenzymes of pig liver esterase, a commercial PLE preparation (Fluka) was separated by 2D gel electrophoresis based on their different isoelectric points and molecular weights (Heymann and Junge 1979a, b; Farb and Jencks 1980). This resulted in 12 different protein spots (Fig. 1). Fractions 1 and 2 were assigned based on their lower p*I* and their higher molecular weight to belong to the γ-isoenzyme. Spots 3 and 4 might correspond to the β-isoenzyme, whereas spots 5, 7, 9, 11 and 12 were assumed to correspond to the α-isoenzyme.

After gel digestion of these protein spots, the peptides were analysed by mass spectrometry. In comparison to the γ-PLE peptide spectrum, significant differences, especially to the putative spectra of the α-isoenzyme, could be found (Fig. 1).

To assign mass peaks to possible amino acid sequences, the known sequence of the γ-PLE was theoretically digested with proteases using the program *peptide mass* http://www.expasy.org/tools/peptide-mass.html). Using this exclusion method, two new peptides were identified in the protein spots of the α-isoenzyme (Fig. 1, circled peaks). These peptides exhibit a molecular mass of 1,286.6 Da ($+H^+$ 1,287.6 Da) and 1,476.79 Da ($+H^+$ 1,477.79 Da) compared to 1,347.6 Da ($+H^+$ 1,348.6 Da) and 1,484.8 Da ($+H^+$ 1,485.8 Da) determined for the γ-isoenzyme. In addition, several conserved regions were also identified, which are summarised in Fig. 2.

Next, tandem mass spectroscopy was applied to clearly identify amino acids differing between the α- and the γ-isoenzyme. This led to the identification of three amino acid substitutions (Q294P, K463N, P302T). Furthermore, two unknown amino acid exchanges in position V236/A237 could be identified. These substitutions were introduced into the gene of recombinant γ-PLE by site-directed mutagenesis. In total, 11 variants (**A** Q294P, **B** K463N, **C** P302T, **D** Q294P/K463N, **E** K463N/P302T, **F** P302T/Q294P, **G** Q294P/K463N/P302T, **W1** V236C/A237I, **W2** V236P/A237G, **W3** A237G, **W4** V236D) were created. The random mutants **W1**–**W4** were introduced by saturation mutagenesis. All variants were transformed to *E. coli* DH5α, sequenced and transformed to *E. coli* Origami (DE3). After 24 h cultivation, the *E. coli* Origami (DE3) cells were harvested

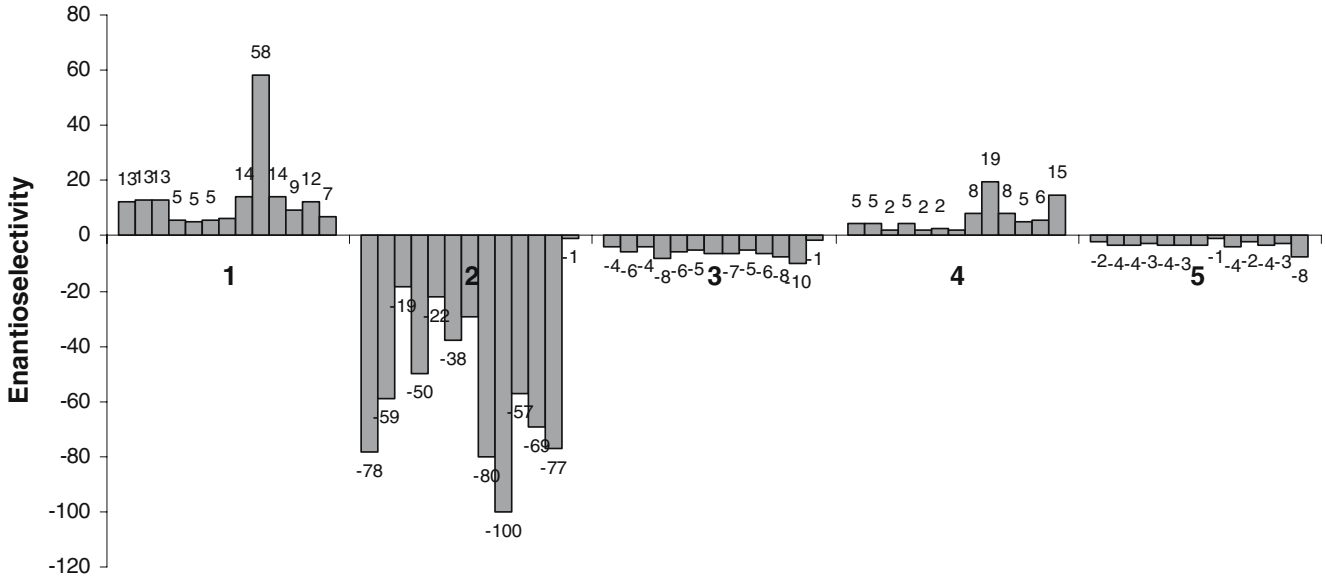

Fig. 4 Enantioselectivities of the PLE mutants A-G, W1-W4, γ-rPLE and Fluka-PLE (from *left* to *right*) towards substrates **1**–**5** as determined by GC analysis; positive *y*-axis: preferentially converted to (*R*)-alcohol; negative *y*-axis: preferentially converted to (*S*)-alcohol

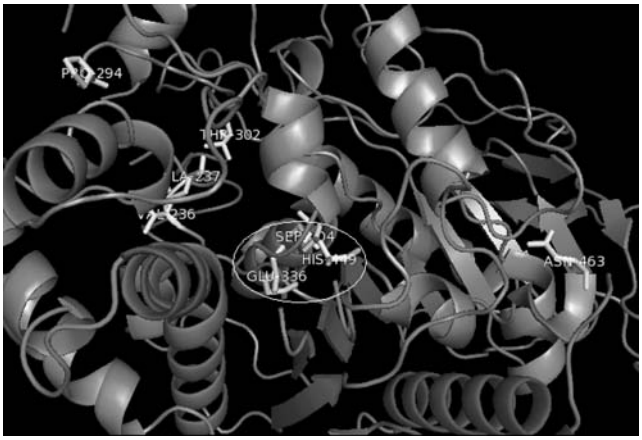

Fig. 5 Homology model of PLE variants based on the structure of human liver carboxylesterase I (Bencharit et al. 2003). The catalytic triad residues (*encircled*) and the mutated amino acids residues are highlighted

by centrifugation, the pellets were washed twice with phosphate buffer (50 mM, pH 7.5) and disrupted by sonification. The supernatants contained the soluble PLE variants exhibiting esterase activity. All PLE variants were His-tag-purified, resulting in enzyme preparations with 4–10 U/ml, corresponding to a specific activity of 20–50 U/mg protein.

The substrate specificities of all variants were investigated towards methyl butyrate, which can be used to distinguish between the α- and the γ-isoenzymes of PLE (Takahashi et al. 1989). Indeed, mutant **F** showed slightly higher conversion of methyl butyrate compared to γ-rPLE (Fig. 3), but none of them was as active as the PLE from Fluka, which is known to contain a large proportion of the α-isoenzyme.

The kinetic resolution of five acetates of secondary alcohols gave that all variants exhibited only small differences in their enantioselectivity towards four substrates. Furthermore, only *E* values lower than 10 could be determined. However, a substantial increase in enantioselectivity was observed for substrates **1**, **2** and **4**. Whereas with γ-rPLE, only an *E* value of 12 towards substrate **1** was determined, reactions with the mutant **W2** (V236P/A237G) reached *E* values of 58, and towards substrate **2**, even an enantioselectivity of E=100 (Fig. 4).

Both mutations V236P/A237G in **W2** enhancing the enantioselectivity of acetate **1** and **2** are situated close to the active site as derived from the homology model of pig liver esterase (Fig. 5).

Discussion

In this study, we aimed to identify the differences in the amino acid sequences of PLE isoenzymes. So far, only the molecular mass, isoelectric points and some indications for the substrate specificities of the various isoenzymes had been reported (Barker and Jencks 1969; Heymann and Junge 1979a, b; Farb and Jencks 1980). To elucidate the amino acid sequences, the isoenzymes of a commercial pig liver esterase sample were separated by 2D gel electrophoresis, digested with proteases, and the resulting peptides were analysed by tandem mass spectrometry analysis. This approach did not allow to determine the complete sequence of the unknown isoenzymes, as mass spectrometry, in general, allows only to determine fragments with a mass ranging from 900 to 3,000 Da and with certain ionisation properties.

However, three amino acid residues (Q294P, K463N and P302T) were clearly identified and assigned to belong to the α-PLE isoenzyme as derived from the molecular mass and

Table 1 Kinetic resolution of (*R*,*S*)-1-phenyl-2-butyl acetate using PLE-mutants **A-G**, **W1-W4,** recombinant γ-PLE, Fluka-PLE und *E. coli* Origami (control)-

PLE variant	Time	Enantiomeric excess		Conversion	*E* value
	(h)	ee_S (%)	ee_P (%)	(%)	
A Q294P	6	58	96	38	−78
B K463N	6	64	94	41	−59
C P302T	6	68	80	46	−19
D Q294P/K463N	6	67	92	42	−50
E K463N/P302T	6	62	84	42	−22
F P302T/Q294P	6	68	90	43	−38
G Q294P/P302T/K463N	6	86	83	51	−29
W1 V236C/A237I	6	95	90	52	−80
W2 V236P/A237G	6	99	93	52	−100
W3 A237G	6	89	90	50	−57
W4 V236D	6	84	93	47	−69
γ-rPLE	8	51	96	35	−77
Fluka-PLE	5	30	3	52	−1.1
E. coli Origami + GroEL/ES	6	1.04	n.d.	n.d.	n.d.

n.d. Not detectable

isoelectric point of the protein spot used for the subsequent tandem mass spectroscopy analysis. Furthermore, two additional positions for an amino acid exchange could be suggested and were used for saturation mutagenesis by QuikChange. All three variants, the corresponding double and triple mutants and the random mutants could be functionally expressed in *E. coli* Origami (DE3) and therefore investigated with respect to their biochemical properties. Unfortunately, no major differences were found in the hydrolysis of methyl butyrate—a substrate preferentially cleaved by the α-isoenzyme. This indicates that further mutations are present in the α-PLE isoenzyme.

However, a significant increase of the enantioselectivity of one mutant towards three different acetates could be obtained. The *E* value found in the conversion of substrate **2** is sufficiently high to enable a practicable kinetic resolution yielding high optical purity (93%ee) for the product enantiomer at 52% conversion. Thus, it could be shown that two single amino acid exchanges are sufficient to change the enantioselectivity of this esterase, which is in accordance to our previous studies dealing with the recombinant pig intestinal carboxyl esterase (Musidlowska-Persson and Bornscheuer 2003a, b).

Furthermore, a homology modelling was performed to find an explanation for the influence of the mutations towards the enantioselectivity. The 3D structure of human liver carboxylesterase I (PDB entry 1MX9A; 77.9% identity to the γ-PLE sequence; Bencharit et al. 2003) was used as template because the structure of PLE has not yet been elucidated. All mutations are situated close to the active site. Valin236 was exchanged to a less flexible proline presumably causing a change in the structure of PLE, resulting in enhanced enantioselectivity towards (*R,S*) 1-phenyl-2-butylacetate and (*R,S*) 1-phenyl-1-ethylacetate. Interestingly, none of the newly introduced mutations had a negative influence on the activity of the variants, as the conversion achieved in the kinetic resolutions (Table 1) were in the same range as for the γ-PLE.

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